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Histochemical demonstration of skeletal muscle fibre types and capillaries on the same transverse section

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Summary. Serial transverse sections of m. vastus lateralis biopsies from six healthy men were reacted:

(1) for myofibrillar adenosine triphosphatase (mATPase) to identify fibre types; or,

(2) with α-amylase, periodic acid–Schiff (αPAS) to visualize capillaries.

Sections were also processed with a new histochemical method for identification of fibre types and capillaries on the same skeletal muscle slice (mATPase/αPAS). Fibre type composition using either method was 41% type I, 37% type IIA and 22% type IIB. Types I, IIA and IIB least diameter areas (mean ± SE, μm^2) were similar in sections processed for mATPase/αPAS or mATPase (3976 ± 338, 5187 ± 373 and 4389 ± 514 vs. 4092 ± 345, 5100 ± 360 and 4289 ± 474, respectively). The number of capillaries per mm^2 and per fibre did not differ in sections processed using the αPAS (315 ± 29 and 1·48 ± 0·12) or mATPase/αPAS (317 ± 25 and 1·43 ± 0·10) method. The number of capillaries was greater ($P < 0·05$) around types I or IIA than type IIB fibres whether a section was processed for mATPase/αPAS (4·5 ± 0·2 or 4·6 ± 0·2 vs. 3·4 ± 0·3) or when fibre profiles of serial sections reacted for mATPase or αPAS were 'matched' (4·5 ± 0·2 or 4·4 ± 0·2 vs. 3·4 ± 0·3). The results indicate that histochemical and morphometric measures can be made on the same transverse section using the new method with substantial savings of time, cost and energy.

Key words: capillarization, fibre area, histochemistry, human muscle, muscle fibre types.

Introduction

Much of our knowledge of skeletal muscle can be attributed to histochemical studies. Different fibre types can be shown in transverse sections reacted for myofibrillar

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adenosine triphosphatase (mATPase) (Brooke & Kaiser, 1970; Guth & Samaha, 1970). Likewise, the capillarity of slow-contracting, high oxidative muscles of lower mammals has been shown to be greater than that of their fast-contracting, low oxidative counterparts (Romanul, 1965; Mai *et al.*, 1970; Gray & Renbin, 1978). These analyses have been relatively simple to perform because muscles or segments of them in lower vertebrates contain predominantly one fibre type (Romanul, 1965; Gray *et al.*, 1983). Analysis of fibre type specific capillarity in human skeletal muscle has been complicated because biopsy samples that contain fast and slow fibres arranged in a mosaic are frequently studied.

The capillarization of specific fibre types in human biopsy samples has been characterized using histochemical (Andersen, 1975; Andersen & Henriksson, 1977; Schantz, 1982, 1983), or combined histochemical and electron microscopy methods (Ingjer, 1977). Adjacent muscle sections were processed to identify fibre types or capillaries, and their fibre profiles matched. However, both methods are extremely labour intensive and data acquisition is an arduous task.

This study describes an alternate method for determining fibre type specific capillarity. The same transverse section of skeletal muscle is processed for fibre types and capillaries using histochemical assays for mATPase and α-amylase, periodic acid–Schiff (αPAS). The results found using the new combined method were compared with those obtained by using the 'matched' fibre profile method of Andersen (1975).

Subjects and methods

SUBJECTS

Six healthy men served as subjects. Their mean (±SE) age, weight, height, and $\dot{V}O_2$ max were 32 ± 4 years, 82 ± 5 kg, 176 ± 3 cm, and 41 ± 1 ml kg^{-1} min^{-1}, respectively. Each provided written consent to participate after the protocol was explained. The study was approved by the Human Research Review Board at the Kennedy Space Center, Florida, USA.

MUSCLE BIOPSIES

Muscle samples were removed from the right m. vastus lateralis using the percutaneous needle biopsy technique (Bergström, 1962) as modified by Evans *et al.* (1982), and processed for histochemical analyses as described previously (Dudley *et al.*, 1983).

HISTOCHEMICAL REACTIONS

Serial transverse sections were processed for:

(1) fibre types (mATPase),
(2) fibre types and capillaries (mATPase/αPAS), or
(3) capillaries (αPAS) using histochemical methods.

The sections for 1 and 2 were first processed for fibre types. Sections for 2 and 3 were then processed for capillaries.

The mATPase reaction used for fibre type expression was essentially as described by Brooke & Kaiser (1970). Sections were pre-incubated at 22°C in an acid (150 mmol l^{-1} sodium barbital, 250 mmol l^{-1} sodium acetate, 100 mmol l^{-1} hydrochloric acid, pH 4·3 or 4·6) or an alkaline buffer solution (100 mmol l^{-1} 2-amino-2-methyl-1 propanol, 18 mmol l^{-1} calcium chloride, pH 10·4).

After acid pre-incubation (7 min) sections were rinsed in 100 mmol l^{-1} tris (hydroxymethyl) aminomethane and 18 mmol l^{-1} calcium chloride (pH 7·8), and transferred to the mATPase incubation solution (100 mmol l^{-1} 2-amino-2 methyl-1-propanol, 18 mmol l^{-1} calcium chloride, 4·5 mmol l^{-1} ATP, pH 9·4) for 15 min at 22°C. Sections from the alkaline pre-incubation (20 min) were transferred directly into the mATPase incubation solution for 15 min.

After incubation, sections were rinsed in 10 g l^{-1} calcium chloride, placed in 20 g l^{-1} cobalt chloride, rinsed in 10 mmol l^{-1} sodium barbital, rinsed in distilled water, incubated in 13 ml l^{-1} ammonium sulphide for 45 s, and finally rinsed in running tap water. The sections intended for the simultaneous demonstration of fibre types (pre-incubation pH 4·6 only) and capillaries were then processed together with untreated sections for visualization of capillaries. The sections intended for fibre type demonstration only (pre-incubation pH 4·3, 4·6 and 10·4) were dehydrated, cleared and mounted on glass slides.

Sections for capillary visualization were incubated for 1 h in 10 g l^{-1} α-amylase at 37°C, rinsed in distilled water and oxidized in 5 g l^{-1} periodic acid. They were then incubated for 20 min in Schiff reagent (Humason, 1979), washed in running tap water, dehydrated, cleared and mounted.

DATA ACQUISITION

Sections were viewed using a light microscope interfaced to an automated image analysis system. Muscle fibres were visually identified and classified as types I, IIA, IIB or IIC (Brooke & Kaiser, 1970). Fibre type proportions are reported as the percentage of the total number of fibres in each section (mean 1082; range 691–1895). Least diameter fibre area was determined from digitized tracings of 50 profiles of each fibre type. Measurements were made using Darwin software (Darwin Instruments, Inc., Winston-Salem, NC, USA). Fibre type specific capillary counts were made from the same 50 fibres of each type used to determine least diameter area. Capillaries were visualized directly from sections reacted for mATPase/αPAS, or by matching fibre profiles in adjacent serial sections reacted for mATPase or αPAS. The 50 values obtained from each method were averaged together to calculate fibre type specific least diameter area and capillary data for each subject. Capillary density and the capillary-to-fibre ratio were determined from a 0·11 mm^2 area of a section reacted for mATPase/αPAS or αPAS.

STATISTICAL ANALYSES

Capillary density and the capillary-to-fibre ratio from the mATPase/αPAS and αPAS methods were compared using a dependent *t* test. A two way (procedure × fibre type) repeated measures analyses of variance was used to analyse least diameter fibre area and fibre type specific capillary data. Differences between means were analysed using Tukey's Studentized Range (HSD) Test. Pearson Correlation Coefficient was used to examine the reliability of data generated by the mATPase/αPAS method compared to values obtained by the mATPase or αPAS method. The significance level was set at $P<0{\cdot}05$.

Results

FIBRE TYPES

Type I, IIA and IIB fibres were readily identifiable in sections processed with the mATPase/αPAS method (Fig. 1). The number of type IIC fibres in any given biopsy was so small that they could not be considered in data acquisition or analyses. The mean (range) fibre type percentages in biopsy samples of m. vastus lateralis were: 41% type I, (16–76%), 37% type IIA, (24–63%) and 22% type IIB, (0–40%).

CAPILLARIES

The sections reacted with the mATPase/αPAS method showed clear contrast between the vivid red capillaries and the variable staining intensities of the muscle fibres (Fig.

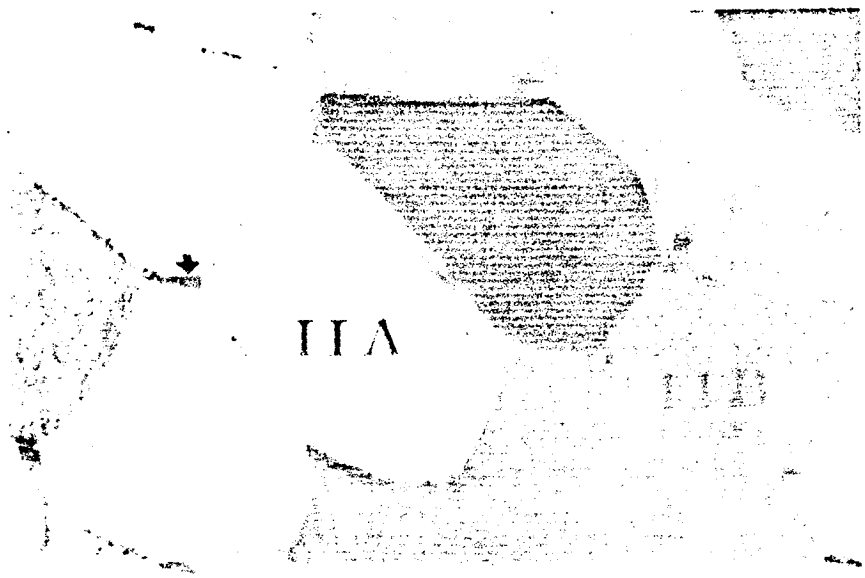

Fig. 1. A serial transverse section of a biopsy sample of the m. vastus lateralis processed with the new combined myofibrillar ATPase/α-amylase, periodic acid–Schiff method following acid pre-incubation (pH 4·6) that shows different fibre types (I, IIA, IIB) and capillaries (arrows) (magnification × 620).

1). The number (mean ± SE) of capillaries per mm^2 was similar for sections processed with the mATPase/αPAS (317 ± 25) or the αPAS (315 ± 29) method. The capillary-to-fibre ratios for the two methods were also similar (1·43 ± 0·10 and 1·48 ± 0·12, respectively). Capillary density and the capillary-to-fibre ratio determined by the two methods were highly correlated ($r^2 = 0·96$ and 0·95, respectively, $P < 0·05$).

The number (mean ± SE) of capillaries around type I or IIA fibres was greater ($P < 0·05$) than the number surrounding type IIB fibres whether sections were processed using the mATPase/αPAS method (4·5 ± 0·2 or 4·6 ± 0·2 vs. 3·4 ± 0·3) or by matching fibre profiles of sections reacted for mATPase or αPAS (4·5 ± 0·2 or 4·4 ± 0·2 vs. 3·4 ± 0·3). The mean values for fibre type specific capillarity were similar for the two methods. The individual values showed a wide range and were highly correlated (Fig. 2a).

FIBRE AREA

The least diameter area (μm^2) of type I, IIA and IIB fibres was similar in sections processed for mATPase/αPAS and for mATPase. The mean values (± SE) were, respectively, type I 3976 (338) and 4092 (345), type IIA 5187 (373) and 5100 (360), and type IIB 4389 (514) and 4289 (474). Type I fibres were smaller than ($P < 0·05$) type IIA fibres. Type IIA fibres were larger than type IIB fibres, but the difference was not significant. Individual values for least diameter area were highly related and showed a wide range (Fig. 2b).

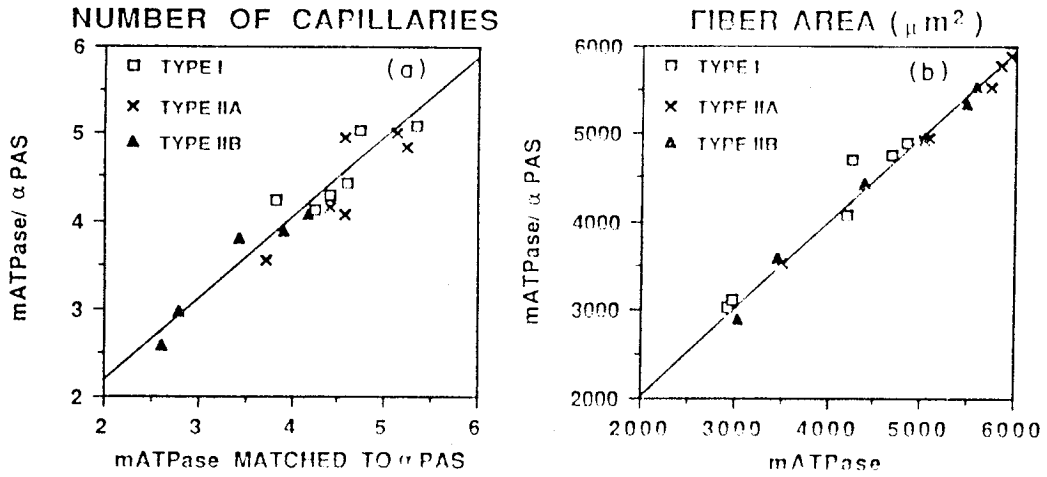

Fig. 2. (a) The relation between the new combined myofibrillar ATPase/α-amylase, periodic acid-Schiff (mATPase/αPAS) and the 'matched' fibre profile (mATPase matched to αPAS) methods to determine the number of capillaries surrounding type I, IIA and IIB fibres. The line is represented by the equation $y = 0·3258 \pm 0·9244\ x$, $r^2 = 0·92$. (b) Comparison between the myofibrillar ATPase (mATPase) and the new combined (mATPase/αPAS) methods for least diameter area of type I, IIA and IIB fibres. The line is represented by the equation $y = 15·10 \pm 0·9806\ x$, $r^2 = 0·99$.

Discussion

This study was designed to establish the efficacy of a new mATPase/αPAS histochemical method for determining fibre type specific capillarity from a single transverse section of skeletal muscle. Morphometric and fibre type specific data were obtained from one transverse section that had been reacted for mATPase and αPAS. These results were compared to those obtained by matching fibre profiles of two adjacent serial sections that had been reacted for fibre types or capillaries using conventional assays for mATPase or αPAS (Andersen, 1975). Six subjects from a pool of 38 were used in this study because their biopsies were known to be highly variable with respect to the area and proportion of the different fibre types. Thus, the validity and reliability of the new 'combined' mATPase/αPAS method were examined over a wide range of morphometric and fibre type specific data.

The capillary supply of muscle has often been reported as capillary density and the capillary-to-fibre ratio. This probably reflects the relative ease with which these measures of capillarity can be made on transverse sections. Our values for these variables are comparable to those reported previously for sedentary subjects (Andersen, 1975; Andersen & Henriksson, 1977). Moreover, the capillary density and the capillary-to-fibre ratio values were highly related when a section was reacted with αPAS or mATPase/αPAS. This indicates that assaying a transverse muscle section for mATPase prior to reaction with αPAS does not alter the capillarity visualized.

The percentage of type I, IIA, IIB and IIC fibres found in the present study are similar to those reported previously for the m. vastus lateralis of sedentary subjects (Andersen, 1975; Andersen & Henriksson, 1977). The relatively uncommon IIAB and IIAC fast-twitch subtypes (Ingjer, 1977; Staron *et al.*, 1983) were difficult to identify because the mATPase reaction product faded during the exposure to periodic acid.

The least diameter area from sections reacted for mATPase or mATPase/αPAS were highly correlated and the average values for each method approximated those reported previously for m. vastus lateralis of untrained subjects (Fig. 2b) (Andersen, 1975; Andersen & Henriksson, 1977). Reaction of transverse sections of skeletal muscle with αPAS has been implied to cause slight enlargement of fibre area (Andersen & Henriksson, 1977). The sections processed using the new combined method in the present study were assayed for mATPase and reacted with αPAS. Their areas for types I, IIA and IIB did not differ by more than 3% from those of sections that had been assayed for mATPase only. Thus, the potential modest enlargement of fibre area in transverse sections of skeletal muscle reacted with αPAS is not evident in the new combined mATPase/αPAS method.

The matched fibre profile (mATPase or αPAS) and the new combined mATPase/αPAS methods gave similar morphometric and histochemical results. As a consequence, they showed comparable fibre type specific capillarity (Fig. 2b). The values obtained in this study are similar to those reported previously (Andersen &

Henriksson, 1977) and show 25% more capillaries around types I or IIA than type IIB fibres.

Andersen & Kroese (1978) have suggested that the capillary supply of a given fibre type should be considered in relation to its area. When the data in the present study are treated in this manner, the new combined and the matched fibre profile methods, respectively, show that the average number of capillaries ($\times 10^{-3}$) per unit area (μm^2) of type I fibres (1·13 and 1·10) is larger than that for type IIA (0·89 and 0·86) or type IIB (0·77 and 0·79) fibres. This method of expression of capillarity may also be of value as different fibre types do not always respond uniformly to alterations in functional demand (Tesch, 1987).

According to the results from this study, the fibre type specific measurements for area, capillarity, and composition are not compromised by the new mATPase/αPAS method. The new combined method reduces tissue handling, requires less tissue, simplifies tissue processing, and reduces cost. By applying the mATPase and αPAS assays to the same muscle section the potential variability caused by making measurements on different sections that are separated by some distance and exposed to different media is removed. Moreover, data acquisition can be performed in a considerably shorter time than when fibre profiles are matched in different sections.

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